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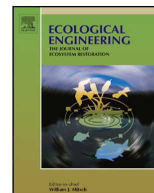

Removal of *Escherichia coli* from synthetic stormwater using mycofiltration

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ABSTRACT

Pathogens from nonpoint sources are the leading cause of water quality impairments in US surface waters. This study assessed the capacity of basidiomycetous fungal mycelium on cellulosic substrates to remove *Escherichia coli* from synthetic stormwater under unsaturated vertical-flow conditions. The mycelium of *Stropharia rugoso-annulata* was tested in mycofiltration columns consisting of 18.6 L containers with mycelium grown on either wood chips or a mixture of wood chips and straw. *S. rugoso-annulata* mycofiltration columns were loaded with water spiked with 600–900 cfu/100 mL of *E. coli* at low (0.5 L/min; 0.57 m/d) and high (2.2 L/min; 2.5 m/d) hydraulic loading. Influent and effluent were monitored for thermotolerant coliform and *E. coli* using the Coliscan membrane filter chromogenic method. Alder wood chips infused with *S. rugoso-annulata* mycelium yielded a removal rate of around 20% relative to control filters. Wood chip and straw media appeared less effective with substantial net export of bacteria from both mycelium-infused and un-inoculated control media. The un-inoculated control media used in this study commonly exported high concentrations of thermotolerant coliform bacteria. On wood chip-based media, the presence of actively growing mycelium reduced the thermotolerant coliform exports by >90% relative to the control media. The study highlights the limitations of using thermotolerant coliform to assess pathogen removal in cellulose rich ecotechnologies like mycofiltration.

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1. Introduction

Microbial pathogens from nonpoint-source discharges such as stormwater are a significant public health concern. Pathogens, including those from nonpoint sources, are the primary cause of surface waters quality impairment in the United States (United States Environmental Protection Agency (USEPA), 2012a). Pathogen-related impairments have substantial societal and environmental costs. Direct contact with fecal contamination can result in adverse health effects such as fever, earache, sore throat, and gastrointestinal illness and poses a particular hazard to swimmers (Haile et al., 1999). Millions of stormwater-attributable waterborne illnesses each year cost hundreds of millions of dollars (Gaffield et al., 2003). Pathogens pose a serious threat to shellfish harvesting, a special concern in Washington State where the commercial shellfish industry is valued at $80 million annually, and where fishing and shellfish license sales and recreational expenses exceed $900 million per year (Booth et al., 2006). Every year tens of thousands of beach closures nationwide cost local communities reliant on tourism and recreation thousands of dollars per day (National Resources Defense Council (NRDC), 2012).

A number of best management practices (BMPs) have been developed to treat pathogens from nonpoint sources. Studies using the International Stormwater BMP database have assessed pathogen removal capabilities of various BMPs (Clary et al., 2008). Few BMPs offer effective removal of fecal indicator bacteria to standards for primary and secondary contact recreation, which are typically around 100–200 cfu/100 mL for thermotolerant coliform. Retention ponds showed some capability of removing bacteria in regions with significant land area and abundant rainfall, but were impractical in urban areas and arid/semi-arid climates due to the lack of space and water. Retention ponds also demonstrated exports of bacteria due to deposits from waterfowl and wildlife. Media filters and biorentention cells also showed some capability of removing bacteria, but were expensive and required regular maintenance. Grass swales and manufactured devices had limited

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Fig. 1. Photograph of natural mycelium of *Stropharia rugoso-annulata*. Mycelium can have surface area per area of ground surface as high as 90 m^2/m^2 (Leake et al., 2004). This high surface area gives fungal mycelium a unique capacity to treat contaminates in the environment. Photo credit: Paul Stamets.

Fig. 2. Photograph of top of mycofilter with *Stropharia rugoso-annulata* on alder wood chips. Bucket was 0.26 m in diameter. Photo credit: Alicia Flatt.

ability to remove bacteria and, similar to retention ponds, often exported bacteria. While treatment wetlands can be sinks for pathogens, they are generally sources of pathogens at low loading (<1000 cfu/100 mL thermotolerant coliform in inflow) (Beutel et al., 2013). Sand filtration is possibly the only BMP that can consistently remove bacterial contamination, but key limitations of the method include low hydraulic loading rate and high maintenance requirements (Bright et al., 2010; Davies and Bavor, 2000). New technologies like porous pavements (Haselbach et al., 2006) and rock biofilters (Beutel and Larson, 2015) have yet to be comprehensively assessed for pathogen removal.

The use of fungi to bioremediate a range of pollutants, while showing promise, has received little attention compared to the use of bacteria (Pinedo-Rivilla et al., 2009). One method of using fungi in bioremediation is mycofiltration—the treatment of contaminated water by percolating it through a network of high-surface-area fungal mycelium (Stamets, 2001, 2005) (Fig. 1). Mycofiltration offers several unique mechanisms for removing bacteria from stormwater, and this diversity of fungal biological processes makes mycofiltration an attractive potential BMP for sustainably reducing pathogens from nonpoint sources. The surface area of fungal mycelium in soil that interacts with the environment can be high. Leake et al. (2004) reported that the length of mycelium in soils ranges from 3 to 600 m/g soil and that the surface area of mycelium in the upper 10 cm of soils ranges from 3 to 90 m^2/m^2 of ground surface area. Fungi are known to produce a wide range of antibiotics and toxins that impair or kill bacteria (Barron, 1992; Barron and Thorn, 1987). Fungi can be predators of bacteria and are able to use bacteria as sources of nitrogen and carbon in nutrient-deficient soils (Fermor and Wood, 1981). Fungi are also capable of rapidly altering metabolism and secondary metabolite production in response to environmental conditions such as osmotic stress (Duran et al., 2010). Some species of fungi also have mechanisms, such as uniquely shaped hyphae, to trap and degrade microbiota within soils (Hong et al., 2006; Swe et al., 2011).

The principal objective of this study was to evaluate the ability of mycofiltration with *Stropharia rugoso-annulata*, commonly known as the “garden giant” or “wine cap” mushroom, to remove *Escherichia coli* from stormwater. *E. coli* was chosen for the study because *E. coli* is a more definitive indicator of fecal contamination in surface waters, and regulatory agencies are shifting away from only using thermotolerant coliform bacteria to assess fecal contamination (United States Environmental Protection Agency (USEPA), 2012b; Arnone and Walling, 2007). Another goal of the project was to assess the potential for biofilters with fungal mycelium grown on cellulosic substrates to release *Klebsiella* sp. bacteria. This is significant because *Klebsiella* sp. bacteria, which test positive for thermotolerant coliform, can come from fecal matter and cellulosic materials including wood pulp and straw (Caplenas and Kanarek, 1984; Mudd et al., 1995). Mycofiltration tests could yield false positives for coliform of fecal origin, or mycofiltration could be mistakenly identified as yielding poor pathogen removal or pathogen production, when only non-specific thermotolerant coliform tests are used to assess contamination.

2. Methods

2.1. Mycofiltration and control media

Biofiltration media and filters were produced by Fungi Perfecti, LLC (Olymipia, Washington, USA). Filters consisted of 18.6 L plastic buckets (height, 0.35 m; surface area, 0.053 m^2) with drain holes filled with 10 kg of mycofiltration or control media (Fig. 2). Two media formulations were prepared, a 100% alder (*Alnus rubra*) wood chip (WC) media and a mixture by weight of 75% alder wood chip and 25% rice straw media (WC/S). The biofiltration media for each formulation was prepared from a single lot of substrate. All mycofiltration media was inoculated with 7.5% by weight pure-culture millet spawn prepared from a single in vitro culture of *S. rugoso-annulata* (non-ATCC indexed strain) maintained by Fungi Perfecti, LLC. Each media type was either inoculated or left un-inoculated as a control and was subdivided into separate growth containers.

In contrast to other mycoremediation studies using solid-state fermentation on pressure-sterilized media, material for this study

was prepared using non-sterile outdoor cultivation so that the data would be more representative of biological performance in an outdoor stormwater treatment setting. Moreover, non-sterile methods are low-tech, inexpensive, and can be readily incorporated into other stormwater management practices such as planted bioretention basins without intensive in vitro cultivation training. The fungal species *S. rugoso-annulata* and two substrate media combinations were selected after numerous growth and vigor trials by Fungi Perfecti, LLC that qualitatively screened six fungal species and five substrates to identify species and substrate combinations likely to withstand environmental conditions found in a stormwater treatment setting. For vigor trials, mycofiltration media was submerged in water for 30 min, followed by incubation for 48 h at 15–20 °C. Media was then re-submerged for 30 min, refrigerated at 3–6 °C for 48 h and frozen at −20 °C for 24 h. Following freezing, the mycofiltration media was stored at 11–16 °C for seven days, followed by warm storage at 25–32 °C for 24 h, followed by hot storage at 32–40 °C for 24 h. Hot storage was followed by a 20 min submersion and six days at 16–17 °C. Additional details on vigor testing can be found in Stamets et al. (2013).

A total of 15 biofilters were produced and tested in 5 treatments in pseudo-triplicate (i.e., triplicates from the same batch of media). Treatments included: (1) vigor-tested *S. rugoso-annulata* on wood chips (VT-SRA-WC); (2) non-vigor-tested *S. rugoso-annulata* on wood chips (SRA-WC); (3) control with wood chips only (CON-WC); (4) non-vigor-tested *S. rugoso-annulata* on wood chips and straw (SRA-WC/S); and (5) control with wood chips and straw (CON-WC/S). Additionally, two SRA-WC/S buckets and two CON-WC/S buckets were produced in the same lot as the other WC/S biofilters and were used for evaluation of resident bacteria species by phylogenetic analysis. Both mycelium-inoculated media (SRA) and un-inoculated control media (CON) were shipped to Washington State University and stored at 13 °C until testing.

2.2. Synthetic stormwater

Synthetic stormwater used in the study consisted of city tap water from the city of Pullman, Washington. The tap water was chlorinated groundwater with moderate hardness and mineral content (total dissolved solids ~200 mg/L). Water was dechlorinated with 25 mg/L of sodium thiosulfate per 30 L of tap water. A stock culture plate of *E. coli* ATCC 11775 was used to inoculate the synthetic stormwater for all trials. For each experimental run, a 5 mL stock solution of *E. coli* was prepared by incubation in a 5 mL vial of Trypticase Soy Broth at 250 rpm and 37 °C for 18 h until the culture reached stationary phase, as determined by consistent cell densities on drop-plate serial dilutions. The stock solution was used to prepare a 1 mL diluted solution with a concentration of ~10^7 cfu/100 mL that was used to inoculate the synthetic stormwater. This produced a 30 L of synthetic stormwater with a calculated *E. coli* concentration of around 700 cfu/100 mL, which was confirmed by monitoring of replicate influent samples (Section 2.6 below).

2.3. Enumeration of resident bacteria populations

Prior to testing, biofilters were acclimated in the laboratory at room temperature (~20 °C) for 48 h. After acclimation and prior to bacteria removal testing, each biofilter was saturated with *E. coli*-free synthetic stormwater. To evaluate the cleanliness of the saturation water, duplicate 100 mL samples were collected prior to biofilter submersion and were analyzed without dilution for *E. coli* and thermotolerant coliform. As each biofilter drained, 100 mL samples of the effluent water were also collected for bacteria analysis.

2.4. Wood chip biofilter testing

The vigor-tested *S. rugoso-annulata* on alder chips (VT-SRA-WC), non-vigor-tested *S. rugoso-annulata* on wood chips (SRA-WC), and control with wood chip (CON-WC) were tested with synthetic stormwater containing ~700 cfu/100 mL *E. coli*. Synthetic stormwater was distributed evenly over the top surface of the biofilter and allowed to percolate vertically through the media at a rate of 0.5 L/min (0.57 m/d). Once outflow started, bacteria samples were collected at 5 and 10 min. The biofilter was allowed to drain for 30 min, and then loaded with 2.2 L/min (2.5 m/d) of percolation solution. Again, samples were collected at 5 and 10 min. Inflow samples were collected at the beginning of each biofilter run at each flow rate for bacteria analysis. Between each set of biofilter tests, all equipment was disassembled, washed, and disinfected with a 10% sodium hypochlorite solution.

2.5. Wood chip and straw biofilter testing

A second test was performed on two sets of three mycofiltration biofilters, one set with non-vigor-tested *S. rugoso-annulata* grown on wood chips and straw (SRA-WC/S) and another set with un-inoculated control wood chip and straw filters (CON-WC/S). The experimental setup followed the procedure for the wood chip biofilter tests described above, except that hydraulic loading rate was decreased to 0.3 L/min (0.34 m/d). For each run, inflow bacteria samples were taken at 5, 15, and 25 min, and outflow bacteria samples were taken at 10, 20, and 30 min.

2.6. Bacteria enumeration

Each bacteria sample was simultaneously monitored for *E. coli* and thermotolerant coliform using the Coliscan C MF method, a U.S. Environmental Protection Agency approved method (9222C) distributed by Micrology Laboratories (Goshen, IN). Enumeration of *E. coli* and thermotolerant coliform followed standard methods (American Public Health Association (APHA), 2005). Samples were collected in sterile bottles and stored at 4 °C and tested within 6 h of collection. Dilution rates varied for different parts of the methods depending on the anticipated level of contamination. Samples of the *E. coli*-free synthetic stormwater used to enumerate resident bacteria populations (Section 2.3 above) were analyzed without dilution due to the very low levels of bacteria present in the tap water. Samples of the biofilter effluent from the initial saturation step (Section 2.3 above) were diluted 1:5 and 1:10 to enable detection from 5 to 1000 cfu/100 mL while maintaining plate counts within detection limits specified by Coliscan (<100 cfu/plate). *E. coli*-spiked synthetic stormwater influent samples were diluted 1:10 and 1:20 to provide plate counts within detection limits specified by Coliscan (<100 cfu/plate). Dilution rates for the effluent samples in the biofilter *E. coli* removal tests (Section 2.4 above) were 1:10 and 1:20 to allow adequate detection at the influent rate and detection up to 2000 cfu/100 mL due to possible resident coliform exports combined with the influent pass-through. For each biological sample, 100 mL duplicates from each dilution were filtered onto 0.45 μm filter pads. Each filter pad was transferred to a petri dish containing an absorbent pad soaked with 1.75 mL of Coliscan MF medium. The dish was incubated inverted at 35 °C for 24 h. The Coliscan chromogenic medium has two color-producing chemicals, one that is activated by the enzyme galactosidase which is produced by general thermotolerant coliforms including *E. coli*, and one that is activated by the enzyme

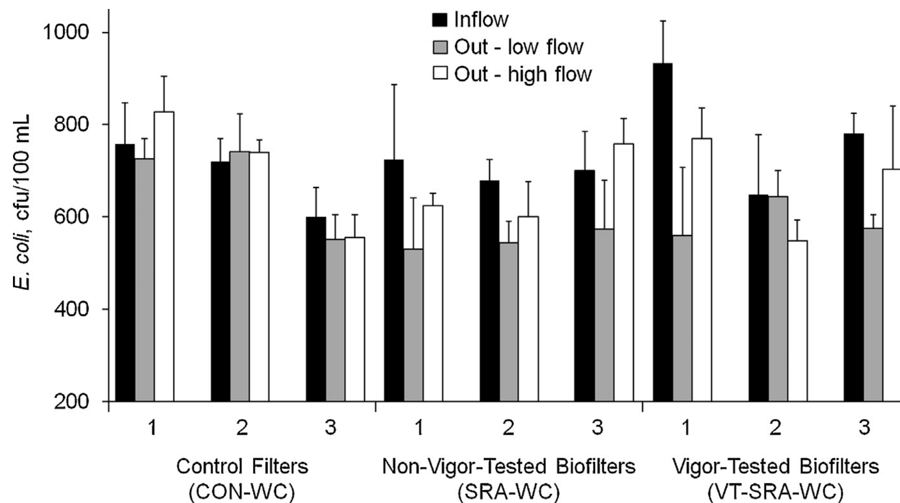

Fig. 3. *E. coli* concentration in inflow and outflow from *Stropharia rugoso-annulata* biofilters. Three treatments performed in triplicate (1, 2, 3) are shown: control filters (CON-WC); non-vigor-tested mycofiltration media (SRA-WC); and vigor-tested mycofiltration media (VT-SRA-WC). Filters were tested under low flow (0.5 L/min, 0.56 m/d) and high flow (2.2 L/min, 2.5 m/d) conditions. Bars are average values and error bars are standard deviation of replicate *E. coli* analyses ($n = 4$).

glucuronidase which is produced primarily by *E. coli*. After the 24-h incubation period, general thermotolerant coliforms appear pink and *E. coli* colonies appear blue/purple. Method blanks were performed after approximately every 10 water samples and no consistent or substantial source of contamination was observed.

Removal effectiveness for *E. coli* was assessed by percent concentration removal (%) calculated as:

$$\frac{\left(\bar{C}_{in} - \bar{C}_{out}\right)}{\bar{C}_{in}} \times 100 \quad (1)$$

where inflow and outflow *E. coli* concentrations were an average of multiple measurements ($n = 4$) made over the 10 min sampling period for the wood chip biofilter test and 30 min sampling period for the wood chip and straw biofilter test.

2.7. Phylogenetic speciation

Colony-forming units from SRA-WC/S and CON-WC/S biofilters were also evaluated for species-level identification of presumptive *E. coli* and thermotolerant coliform colonies. According to procedures described for the experimental setup, without the addition of *E. coli* stock solution to the influent water, two SRA-WC/S and two CON-WC/S biofilters that had not been tested in the synthetic stormwater protocol were flushed with synthetic stormwater for 30 min at a rate of 0.3 L/min (0.34 m/d). Samples were collected at 10, 20, and 30 min, diluted 1:10, 1:20, and 1:100 to clearly separate CFUs, and were plated using the Coliscan MF method. Two pink CFUs and seven blue CFUs from replicate biofilters were selected based on clear separation from other colonies and were subcultured from the Coliscan plate onto Brain Heart Infusion (BHI) agar. BHI plates were incubated for 24 h, inspected to ensure consistent growth without visible contamination, and were sent for phylogenetic analysis to the bacteriology identification laboratory Microcheck (Northfield, Vermont, USA).

3. Results

3.1. Resident bacteria in biofilters

When initially saturated with synthetic stormwater free of *E. coli*, the resulting effluent from all biofilters exported bacteria that tested positive as thermotolerant coliform, although at widely differing levels. Biofilter media that contained straw consistently exported thermotolerant coliform bacteria at levels that were too high to enumerate at a 1:20 dilution rate, indicating that concentrations in the effluent exceeded 2000 cfu/100 mL. Wood chip biofilter media also exported thermotolerant coliform bacteria, but at substantially lower levels than in media that contained straw. Exports were lowest from the SRA-WC mycofiltration media, with effluent averaging 30 cfu/100 mL. Vigor tested mycofiltration media (VT-SRA-WC) exported moderaterately higher thermotolerant coliform levels, with effluent levels averaging 133 cfu/100 mL. Exports from the corresponding wood chip control media (CON-WC) averaged 672 cfu/100 mL.

Large exports of bacteria that tested positive as *E. coli* were also observed in all biofilter media that contained straw, with concentrations consistently exceeding 2000 cfu/100 mL. In contrast, no *E. coli* were observed in outflow from SRA-WC mycofiltration media, and comparably low levels (<40 cfu/100 mL) were observed in outflow from the VT-SRA-WC mycofiltration media and the CON-WC control.

3.2. *E. coli* removal by biofilters

Alder wood chips infused with *S. rugoso-annulata* mycelium demonstrated the capacity to remove *E. coli* from synthetic stormwater while control wood chip filters showed only minimal removal (Fig. 3). Both VT-SRA-WC and SRA-WC mycofiltration media exhibited improved removal relative to control filters at low hydraulic loading. Under high hydraulic loading, mycofiltration appeared less effective with removal rates similar to the controls. Percent concentration removal averaged from −3 to 2% in CON-WC filters and 4–22% in *S. rugoso-annulata* mycofiltration media (Table 1). Results from the wood chip and straw biofilters (WC/S media) were less conclusive, with most filters showing large levels of *E. coli* export with average percent concentration removals in excess of −100% (Table 1).

3.3. Thermotolerant coliform export by biofilters

Monitoring of thermotolerant coliform exports by biofiltration media during the *E. coli*-spiked synthetic stormwater filtration tests indicated that all control media types exported some thermotolerant coliform bacteria, but suggested that SRA-WC media decreased

Table 1
E. coli percent concentration removal in biofilters.

Experimental treatment	Low flow rate	High flow rate
Wood chip biofilter testing		
(1) Vigor-tested *Stropharia* on wood chips (VT-SRA-WC)	22 ± 11	14 ± 2[b]
(2) *Stropharia* on wood chips (SRA-WC)	22 ± 3[a]	4 ± 6
(3) Wood chip control (CON-WC)	3 ± 3	−2 ± 5
Wood chip and straw biofilter testing		
(4) *Stropharia* on wood chips and straw (SRA-WC/S)	−295 ± 24[a]	
(5) Wood chip and straw control (CON-WC/S)	−56 ± 24	

Negative value indicates export of *E. coli* from biofilter. Values are average plus/minus standard error for triplicate biofilters.
[a] Removal was significantly different from control (two-tail Student *T*-test; $p < 0.05$).
[b] Removal was nearly significantly different from control (two-tail Student *T*-test; $p = 0.054$).

thermotolerant coliform exports by >90% relative to controls at both low and high flow rates (Fig. 4). Of the wood-based media types, the CON-WC exported the highest concentrations of thermotolerant coliform bacteria, averaging 261–565 cfu/100 mL. The VT-SRA-WC media exported thermotolerant coliform bacteria at a modestly lower rate than the controls, with average exports ranging from 14 to 308 cfu/100 mL. The SRA-WC mycofiltration media exported near zero levels of thermotolerant coliform bacteria relative to the CON-WC media controls, with exports averaging between 0 and 3 cfu/100 mL. On wood and straw based media, both the SRA-WC/S mycofiltration media and the CON-WC/S controls exported high levels of thermotolerant coliform bacteria, with exports consistently exceeding 2000 cfu/100 mL.

3.4. Phylogenetic speciation

Nine colony forming units isolated from the effluent of two SRA-WC/S biofilters and two CON-WC/S biofilters were tested for phylogenetic speciation (Table 2). Six of these plates were analyzed in duplicate as a control for the purity of the sub-culture and the reliability of gene sequence alignments. All duplicates showed a high degree of uniformity. As a positive control for the phylogenetic methods, a plate of the stock culture of *E. coli* ATCC 11775 was also sent for genetic identification, and was confirmed. Two pink thermotolerant coliform colonies from pseudo-replicate SRA-WC/S mycofiltration media buckets were identified as *Raoultella planticola*. Seven presumptive *E. coli* colonies from both the SRA-WC/S and CON-WC/S media types could not be confirmed as *E. coli* using genetic methods. These blue-staining colonies appeared to be predominantly *Enterobacter* sp. and *R. planticola*.

4. Discussion

4.1. *E. coli* removal in biofilters

The most unequivocal result of this study was the ~20% decrease in *E. coli* concentration observed in *S. rugoso-annulata* mycofiltration media on a 100% alder wood chip substrate (Table 1). Compared to control filters, removal was significantly higher ($p < 0.05$, two-tailed Student *T*-test) in the SRA-WC biofilters. In addition, *E. coli* removal was observed in VT-SRA-WC biofilters, indicating that mycofiltration media may be capable of *E. coli* removal even when exposed to strenuous field conditions. *E. coli* removal by the VT-SRA-WC media was more variable, however, possibly due to exports of resident bacteria. This study also helps to constrain the range of acceptable areal hydraulic loading for mycofiltration. Consistent *E. coli* removal was observed at loading rates on the order of 0.3 to 0.6 m/d.

There are only a handful of studies that have evaluated bacteria removal using mycofiltration, and removal dynamics were similar to rates seen in this study. Rogers (2012) performed a lab-scale mycofiltration experiment using columns (20 mm diameter, 200 mm length) packed with alder sawdust inoculated with *Pleurotus ostreatus*. The columns were loaded with water with an *E. coli* concentration of ~10^7 cfu/100 mL. Hydraulic loading rates were low and ranged from 2 to 20 mL/min. Average *E. coli* concentration removal rates were around 20%. Thomas et al. (2009) performed a field-scale mycoremediation study in the Dungeness Watershed in western Washington. Two vertical-flow biofilters (18 m^2 area, 1.5 m thick) were constructed with layers of sandy loam, organic compost, geotextile fabric, and gravel. One biofilter

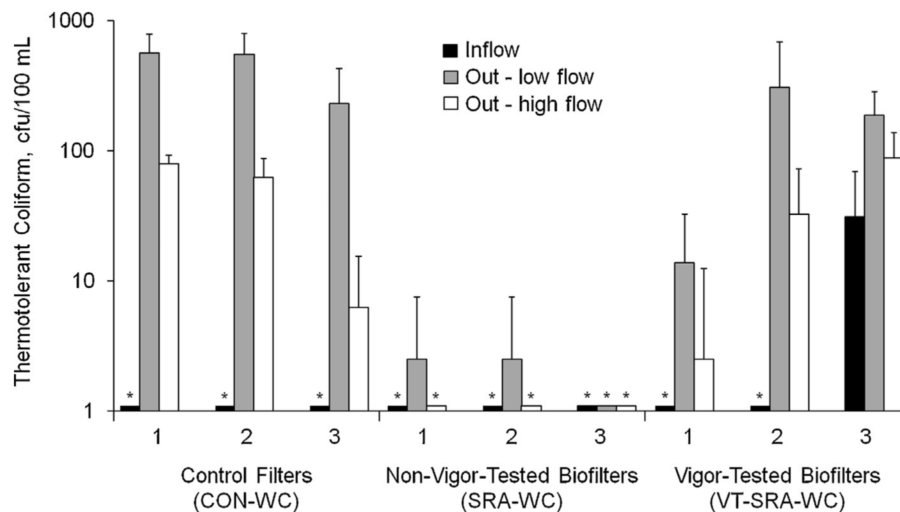

Fig. 4. Thermotolerant coliform concentration in inflow and outflow from *Stropharia rugoso-annulata* biofilters. Three treatments performed in triplicate (1, 2, 3) are shown: control filters (CON-WC); non-vigor-tested mycofiltration media (SRA-WC); and vigor-tested mycofiltration media (VT-SRA-WC). Filters were tested under low flow (0.5 L/min, 0.56 m/d) and high flow (2.2 L/min, 2.5 m/d) conditions. Bars are average values and error bars are standard deviation of replicate thermotolerant coliform analyses ($n = 4$). Bars with an asterisk indicate that no thermotolerant coliform bacteria were detected.

Table 2
Phylogenetic Identification of Colonies in Biofilter Outflow.

Biofilter	Color on Coliscan MF plate[a]	Replication of genetic testing	Identification[b]	(%) Match
E. coli control	Na	Single	*Escherichia coli* (ATCC 11775)	99.9
SRA-WC	Pink	Single	*Raoultella planticola* (ATCC 33558)	100
SRA-WC	Pink	Single	*Raoultella planticola* (ATCC 33558)	100
SRA-WC	Blue	Single	*Staphylococcus hominis hominis* (ATCC 27844)	100
SRA-WC	Blue	Duplicate	*Enterobacter aerogenes*	99.9/99.9
SRA-WC	Blue	Duplicate	*Enterobacter aerogenes*	99.9/99.9
SRA-WC	Blue	Duplicate	*Enterobacter aerogenes*	99.9/99.9
SRA-WC	Blue	Duplicate	*Raoultella planticola* (ATCC 33558)	100/99.9
CON-WC	Blue	Duplicate	*Enterobacter* sp.	96.6/96.7
CON-WC	Blue	Duplicate	*Raoultella planticola* (ATCC 33558)	99.7/100

na—Not applicable.
[a] Pink color indicates thermotolerant coliform; blue color indicates *E. coli*.
[b] ATCC—American Type Culture Collection.

was inoculated with the mycelia of *S. rugoso-annualata* and *P. ostreatus*. A second biofilter contained wood chip mulch without fungal inoculum. Biofilters were lightly loaded at approximately 1.4 L/min (0.003 m/d) with mildly contaminated inflow from a lagoon (<40 cfu/100 mL). The study reported a 66% reduction in thermotolerant coliform in the control filter and a 90% reduction in fungal inoculated biofilter. Chirnside et al. (2013) examined the effectiveness of *P. ostreatus* in a spent mushroom compost in reducing *E. coli* in simulated stormwater runoff. They found that the number of *E. coli* increased exponentially in control reactors but decreased in reactors with the spent mushroom compost.

4.2. Thermotolerant coliform export from biofilters

In a number of test runs and particularly in the WC/S trials, outflow from mycofiltration and control filters tested positive for thermotolerant coliform, even though monitoring confirmed that inflow contained only *E. coli.* To identify these microorganisms, pink colonies grown on Coliscan plates from SRA-WC/S and CON-WC/S biofilters were sub-cultured and sent for phylogenetic analysis to the bacteriology identification laboratory Microcheck (Northfield, VT). Pink colonies were identified as *R. planticola* (Table 2). This bacterial species was formerly grouped into the genus *Klebsiella* until it was reclassified into the new genus *Raoultella* (Drancourt et al., 2001). While the data is preliminary, these phylogenetic results support the contention that biofiltration media can export *Klebsiella* sp. that test positive for thermotolerant coliform, even though the source of the bacteria may have been cellulosic material in the biofilter and not fecal contamination (Caplenas and Kanarek, 1984; Mudd et al., 1995).

Since high levels of thermotolerant coliform were consistently observed in outflow from biofilters with wood chips and straw, with lower levels emanating from biofilters with only wood chips, this study suggests that straw in the biofilters supported the growth of these organisms to a greater extent than the wood-based media. While this contention is somewhat controversial due to the ubiquitous use of straw in stormwater management, straw as a source of thermotolerant coliform contamination has been documented in watershed source-tracking studies (Mudd et al., 1995). Notably, the wood chip media infused with actively growing *S. rugoso-annulata* mycelium (SRA-WC) did not export thermotolerant coliform bacteria and the stressed mycelium (VT-SRA-WC) exported lower levels than the control. This suggests that the growth of *S. rugoso-annulata* on wood chips may create an environment that is less suitable for the growth of thermotolerant coliform type bacteria than wood chips that are not growing this fungal species.

There are many possible mechanisms for this phenomenon, which have been comprehensively reviewed (De Boer et al., 2005). One plausible explanation for the apparent antagonism on a wood chip substrate and apparent commensal relationship on a wood and straw substrate is that *S. rugoso-annulata* is able to out-compete the cellulose-decomposing thermotolerant coliform bacteria such as *Raoultella* (*Klebsiella*) on a wood substrate. The cellulose in wood exists in a tightly-bound crystalline (fibrillose) structure embedded in a matrix of hemicellulose and lignin that is resistant to bacterial enzymatic hydrolysis, but is accessible to fungi with lignin-degrading enzymes. In the media blend that contained straw, the increased cellulose availability provided by the straw may have removed this selective pressure. The identification of non-fecal source *Raoultella* (*Klebsiella*) sp. in both mycofiltration and un-inoculated biofilter outflow highlights the limitation of using thermotolerant coliform to assess removal of pathogen indicator species in cellulose-rich ecotechnologies like mycofiltration.

4.3. *E. coli* export from biofilters

Another ramification of the presence of *Klebsiella* sp. is that the organisms may have led to false positives for *E. coli* on the chromogenic media used in this study. Large exports of both thermotolerant coliform and bacteria that tested positive as *E. coli* were observed in all biofilter media that contained straw. A few studies have reported that some common non-*E. coli* thermotolerant coliform, including members of the *Klebsiella* genus as well as *Enterobacter aerogenes*, can produce the key enzymes, galactosidase and glucuronidase, that result in a positive *E. coli* detection on membrane filtration chromogenic media methods such as the Coliscan system (McLain et al., 2011; Alonso et al., 1999; Kämpfer et al., 1991). The non-specificity of these enzymatic detection systems was observed in this study as well, with *Raoultella* (*Klebsiella*) *planticola* and *E. aerogenes* accounting for roughly 70% of the presumptive *E. coli* colonies that were analyzed with genetic methods (Table 2). However, false positive rates for *E. coli* detection on chromogenic media are also known to be exacerbated by crowded plates (McLain et al., 2011; Olstadt et al., 2007), which were common in the WC/S trials because of large apparent export of *Raoultella* (*Klebsiella*) *planticola* and *E. aerogenes* from biofilters without accompanying increases in serial dilution during monitoring. False positives induced by high levels of these bacteria at high concentrations on the Coliscan plates may partially explain the large *E. coli* levels observed in outflow from the WC/S biofilters. In the wood-based media trials, no *E. coli* were observed in outflow from SRA-WC mycofiltration media, however, low levels were observed in outflow from the VT-SRA-WC media. This monitoring indicated that there was not a native population of *E. coli* or false-positive-*E. coli* growing on the SRA-WC media; however, there may have been some export of resident *E. coli* or false-positive *E. coli* from the VT-SRA-WC media. This may partially explain the higher variability in

E. coli removal rates seen in the VT-SRA-WC media relative to the SRA-WC media.

4.4. Recommendations for future research

Analogous to the early days of developing constructed treatment wetlands using aquatic plants, interest in fungal-based biofiltration systems is growing (Stamets, 2005; Taylor and Stamets, 2014). The concept of utilizing cultivated fungi to perform environmental services in water treatment applications warrants future research to realize its potential as a new ecological engineering technique. While the results of the present study are preliminary, the use of *S. rugoso-annulata* mycelium as an amendment for improving *E. coli* removal by wood-based biofiltration media appears promising. To allow for a more complete interpretation of biological monitoring data, future efforts to assess pathogen removal by mycofiltration should include multiple approaches for microbial identification in biofilter outflow, including chromogenic media, plating, and phylogenetic analysis. Thorough analysis of any correlation between *E. coli* indicator bacteria removal and waterborne pathogen removal will also be essential to fully understand the value of mycofiltration and to support its adoption as a regulated management practice. Future efforts would also be improved by comparing *E. coli* removal between material produced using sterile and non-sterile methods. While sterile methods are not likely to be applicable or practicable in the field, a comparison between these methods would illuminate possible confounding variables in this study, such as a resident population of coliform bacteria on some substrate types. Additional research is also needed to explore straw as a potential source of thermotolerant coliform bacterial contamination. This research is of particular regulatory significance due to the widespread use of straw as a stormwater treatment practice and the growing number of bacteria total maximum daily load (TMDL) regulatory actions nation-wide. Further investigations using straw from multiple sources could also help determine if straw as a substrate is susceptible to harboring thermotolerant coliform bacteria, as suggested by this study.

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